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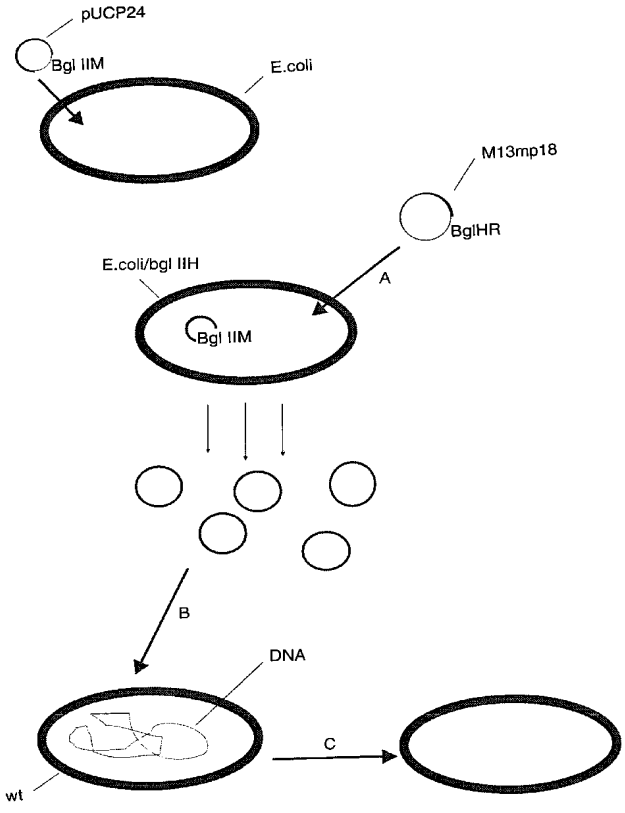
(54) Title: MODIFIED PHAGE, COMPRISING A NON-LYTIC MODIFICATION AND EXPRESSING A KIL-GENE



(57) Abstract: A phage is provided whereby its lysis cassette comprises a non-lytic modification and a non essential region of its genome comprises at least one kil-gene as well as a method for producing such a phage, a phage DNA, an antibacterial agent and a pharmaceutical preparation comprising the phage.

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MODIFIED PHAGE, COMPRISING A NON-LYTIC MODIFICATION AND EXPRESSING A KIL-GENE

The present invention relates to a modified phage, a modified phage DNA as well as a method for producing the modified phage.

The appearance of bacteria resistant against commonly used antibiotics has become a serious threat in medical centers. Nosocomial infections acquired particularly by immunsuppressed patients in hospitals are steadily increasing. An alternative to chemical antimicrobial agents to treat acute bacterial infections is the use of bacteriophages specific for a given bacterial pathogen. Phage therapy has been extensively used in the former Soviet Union and in Poland. An advantage of phage therapy over chemotherapy appears to be the specific targeting of the phage to the pathogen without affecting the normal microbial flora of the body. These studies hold promise that phage therapy under the right conditions, is as effective as chemotherapy.

Bacteriophages which were developed in order to be used in therapy of bacterial infections are described in the US 5 811 093. According to this document bacteriophages are developed by serial passage or by genetic engineering to obtain bacteriophages capable of delaying inactivation by any component of the animal's host defense system against foreign bodies. These phages were able to survive for longer periods of time in the circulation and the tissues than the wild-type phage.

In the US 6 121 036 a bacteriophage preparation is described whereby the preparation consists essentially of two or more bacteriophages and these bacteriophages are isolated against different strains of bacterial organisms. The US 5 366 882 A relates to isolated DNA coding for a restriction endonuclease and/or methylase in order to provide a system for the use in various genetic engineering methods. The SU 1 463 759 A discloses a plasmid comprising a gene for an active rexAB which limits the growth of the natural forms of T4, T5 and T7 bacteria phages as well as defective genes of repressors cI and cro which suppress rexAB expression. These plasmides increase the resistance of E.coli cells to various bacteria phages.

The SU 1 130 602 A relates to a recombinant plasmid DNA which determines the synthesis of endonuclease of restriction Eco RV.

The WO 92/01791 A1 relates to a modified bacterium comprising the gene of a lytically active membrane protein from bacteriophages or a gene of a lytically active toxin-release gene in order to produce bacteria ghosts which can be used as immunogenes.

The EP 0 278 697 A2 discloses an expression system comprising a kil gene which will be used to release paraplasmic proteins into the culture medium in order to produce proteins of medical and industrial importance.

However, in all the described phage therapies the phages induced host cell lysis in Gram+ and Gram- bacteria which results in a massive release of cytoplasmic and cell wall components including endotoxin or superantigenic exotoxin, lipoteichoic acids and muropeptides of bacteria. These substances produce in the organism treated with the phages undesired side effects which can result in symptoms which are even worse than the symptoms resulting from the bacterial infection.

The object of the present invention is therefore, to provide phages which can be used in phage therapy to effectively kill specific bacteria and simultaneously avoiding undesired side effects, e.g. release of cell debris. The object of the present invention is to provide phages which are safe and will not cause any harm to the organism to which they are administered, which will, however, be effective in killing any strain of bacteria and therefore also kill bacteria which are resistant to antibiotics. Thereby the object of the present invention is to provide phages which can replace antibiotics in antibacterial therapy.

The object of the present invention is achieved by a phage characterized in that its lysis cassette comprises a non-lytic modification and a non essential region of its genome comprises at least one kil-gene.

In the scope of the present invention the term "lysis cassette" is used for the sequence in the phage genome which is necessary

for cell lysis upon phage infection. In general bacteriophages which comprise a lysis cassette are either virulent phages or temperent phages, e.g. phages which have the ability to carry out a lytic cyclus once they have infected the bacterial cells. The lysis cassette therefore comprises in particular genes coding for proteins necessary to cause cell lysis. However, the lysis cassette according to the present invention also comprises regions of the phage genome which are able to regulate the expression and activity, respectively, of these genes the products of which are capable of causing cell lysis.

Except for filamentous phages, all bacteriophages require frank lysis of the host. In general, the strategy of lysis is different for large, complex phages and small, simple phages. Large phages (i.e. λ, T7, T4) elaborate a peptidoglycan-degrading enzyme activity which can be detected in the lysate, whereas small phages (i.e. ØX174 and MS2) generally do not and instead accomplish host lysis by other means. Traditionally, endolysin is the term assigned to the peptidoglycan-degrading activity found in the lysates of large phages, even though there are a variety of different enzymes which serve this purpose (Young et al., FEMS Microbiology Reviews 17 (1995), 191-205).

Therefore, such "lysis cassettes" may comprise but are not limited to lysozymes, transglycosylases, endopeptidases, amidases (see Young R. (1992) Bacteriophage lysis: mechanism and regulation. Microbiol. Rev.56, 430-481) as well as hol-genes, lysis gene L of the male-specific RNA phage MS2, A_2 maturation protein from group III RNA phage Qβ, lysis gene E of single stranded DNA phage ØX174 and others. These genes and lysis systems have been well characterised and the person skilled in the art will be able to define the lysis cassette of a given phage without undue burden.

The term "non-lytic modification" in the scope of the present invention comprises any modification in the above mentioned lysis cassette which modification renders a phage comprising a lysis cassette non-lytic. A phage with a non-lytic modification in its lysis cassette does not cause cell lysis once it has infected a bacterial cell. This non-lytic modification comprises in particu-

lar mutations, e.g. deletions, substitutions, additions which render the lysis cassette of the phage non active meaning that a phage comprising a non-lytic modification is able to infect bacterial cells, however, it will not cause cell lysis. These modifications may comprise imitations in a specific lysis gene. However, also modifications which have a regulatory level are comprised in the scope of the present application. Thus, phages with non-lytic modifications propagate in the bacterial cell. However, no substances will be set free and no side effects will occur which is the usual result when phages with lysis cassettes infect bacteria (see Bläsi U. and Young R. (1996), Molec.Microbiol. 21:675-682; Young R. and Bläsi U. (1995), FEMS Microbiol. Rev. 17:191-205).

The term "kil-gene" in the scope of the present invention comprises any gene which will cause a loss of viability to the bacterial cell in which the phage is propagated. The kil-gene will for example prevent the action, function, expression, translation or transcription of a certain protein necessary for the bacterial cell. The kil-gene may for example code for a protein which interrupts one or more steps of the cell metabolism, proteins which bind to specific regions of the DNA or proteins of the bacterial cell thereby inhibiting the DNA or proteins necessary for the normal functioning of the cell and others. The main characteristic of the kil-gene is that it causes a loss of viability to the cell in which the phages are propagated, i.e. cell death. Generally it is sufficient if the phage genome comrises only one kil-gene. However it is of course possible that the genome comprises two or more genes that may interact with each other or attack the cell at different places thereby increasing the safety of the phage therapy with a phage according to the present invention. Preferably the kil-gene is a gene which does not naturally occur in the phage.

However, it is important that the phage according to the present invention is able to propagate in a system in order to produce a sufficient quantity of the inventive phage. One possibility is that the kil-gene is part of a toxin-antitoxin system, whereby in the host cells, in which the inventive phages propagate the kil-gene is not active. Such systems are well known in the art and

always comprise two components: A toxin and antidote which prevents the lethal action of the toxin. Often the toxin is stable and the antidote is unstable, whereby the antidotes often prevent the lethal action of the cognate toxins by forming tight complexes with them. Two different types of post-segragational killing systems are known in bacteria: (i) those in which the antidotes are proteins (the proteic killer gene systems) and (ii) those in which they are antisense RNAs (Jensen et al. Molecular Microbiology (1995) 17 (2), 205-210). An example of the second group (ii) is the hok/sok system: Hok mRNA encodes the Hok killer protein of 52 amino acid residues. Expression of hok is regulated post-transcriptionally by Sok antisense RNA (see Thisted et al., J. Mol. Biol.(1992) 223, 41-54 which is incorporated herein by reference). In this type of mechanism, the regulators are unstable antisense-RNAs that inhibit the translation of stable toxin-encoding mRNAs. The instability of the antisense RNAs leads to activation of translation of the toxin encoding mRNAs (see Gerdes et al. Journal of Bacteriology, Feb. 2000, 561-572 which is incorporated herein by reference).

The first group (i), in which the antidotes are proteins, comprises as antidotes proteins whereby the toxin-antitoxin are often two proteins encoded by adjacent genes located in an operon. The operons are often autoregulated at a level of transcription either by a complex formed by the toxins and the cognate antidotes or by the antidotes alone. In these systems a stable toxin and an unstable antidote are encoded whereby the antidotes prevent the lethal action of the cognate toxins by forming tight complexes with them. The antidotes are degraded by cellular proteases. Such a system may be but is not limited to: ccd of F, parD/pem of R1/R100, parDE of RP4/RK2, and phd/doc of P1. The person skilled in the art will be able to find and select an optimal toxin/antitoxin system without undue burden as shown for example in Mittenhuber (J. Mol. Microbiol. Biotechnol (1999) 1(2): 295-302) where mazEF like antitoxin/toxin systems were found which article is incorporated herein by reference.

Even though such toxin/antitoxin systems are particularly advantageous since they allow on the one hand a phage which will kill the target cells and on the other hand propagation in host cells

without killing, of course any other system can be used. For example a system in which the kil-gene is selected to kill selected bacteria, however, it will not kill the host cells in which it will be propagated.

A further example of a kil-gene is the holin-gene: The function of phage encoded holins is to punch a hole in the inner membrane which leads to efficient cell killing without an immediate disintegration of the cell. As thus far known, holins of different phages are active in Gram-negative as well as in Gram-positive bacteria. The propagation of the phage comprising a holin gene as a kil-gene may be carried out in bacteria which comprise antisense RNA in order to completely silence the holin gene during propagation of the modified phage (see Bläsi et al. FEMS Microbiol. Rev. 17 (1995) 191-205 which is incorporated herein by reference).

Further possibility of a kil-gene relates to type II restriction/modification systems. The action of the restriction endonuclease is prevented in the host used for propagation of the modified phage through modification of the substrate by a corresponding methylase.

However, it is important that the kil-gene is comprised in a non-essential region of the phage genome. According to the present invention a "non-essential region" is a sequence of the genome which is not essential for the phage which means that a phage comprising the kil-gene is still able to infect cells and propagate normally. The person skilled in the art will be able to detect and select such a non-essential region in a phage genome without undue burden since such non-essential regions are already described in the literature and have been used for insertion of foreign genes and a person skilled in the art will be able to find further non-essential regions in a given phage.

The phage according to the present invention is therefore able to infect bacterial cells, propagate within these cells and cause a loss of viability to the bacterial cells, however, without causing cell lysis. Therefore, phages are provided which kill bacteria, however, without the usual side effects caused by cell

debris. These phages are therefore efficient and safe for therapeutical use.

Of course any phage which is able to cause cell lysis can be modified according to the present invention. The phage may be selected according to the specific bacteria to be killed. However, it is also possible to modify one phage in such a way that this phage will specifically recognize a number of bacteria, e.g. by for example introducing in its genome a gene which will provide the ability to the phage to recognize for example a specific antigene, surface protein, bacterial pili, LPS of Gram-negative bacteria, techoic acids of Gram-positiv bacteria, etc.

By using a peptide library of one or more bacteria strains phages with a broad host strain spectrum can be provided. In particular such which can infect the majority of clinical isolates, e.g. of Staphylococcus aureus isolates. Thereby, the problem of phage resistant bacterial mutants can be solved.

Preferably, the non-lytic modification in the cell lysis cassette comprises an expression-disrupting modification in a holin gene. The holin gene is the gene coding for the holin protein which is a small protein which couses a non-specific lesion in the cytoplasmic mebrane which allows transit of the endolysin (see below) to the cell wall which is accompanied by cell lysis. The holin gene is therefore one of the main factors contributing to cell lysis. By entering an expression-disrupting modification in the holin gene the expressed holin protein is either modified in such a way to render it inactive or the holin protein is not expressed at all. Again the modification may be any mutation in the gene or in a DNA region necessary for the holin-regulation or in the above mentioned region, but also a deletion of part of the gene or of the complete gene. Phages with an expression-disrupting modification in the holin gene do not cause lesions in the cytoplasmic membrane of the bacteria. Thereby, such phages are not able to cause cell lysis.

Three types of functional holins have been described: type I (λ S, P22 gp13, P2 Y, P1 LydA, PRD1 ORFM) which are 87 residues or longer and contain three or more regions which might be consid-

ered as membrane-spanning domains; type II (21 S, PA-2 S, Ø80 S, T7 gp17,5, T3 Lys, and Hp1 ORF78) which are 78 residues or smaller and have only two potential membrane-spanning domains, and a third type, solely occupied by T4 t, which does not resemble any other holin sequence. Within these types, there are gene families which have clear evolutionary relationships discernible from sequence similarity (FEMS Microbiology Reviews 17 (1995) 191-205). Of course, any other here not mentioned holin-gene will be comprised by the present application.

A further preferred embodiment of the phage according to the present invention is characterized in that the non-lytic modification in the cell lysis cassette comprises an expression-disrupting modification in an endolysin gene. The endolysin is a protein whose substrate is the peptidoglycan of the bacteria. Also here the same as described for the holin gene is applied: By entering an expression-disrupting modification in the endolysin gene of the phage the phage is unable to lyse the cell.

To date at least four different kinds of enzymes have been identified as filling the endolysin role for different bacteriophages; lysozymes, transglycosylases, endopeptidases and amidases. The T4 lysozyme has been shown to be a soluble, monomeric 15-kDa protein with a basic isoelectric point. Because of the absence of a signal sequence, T4 lysozyme, and the other endolysins as well, accumulate in an active, fully folded form in the cytoplasm during the vegetative cycle. At the end of the vegetative cycle, and in fact defining the end of the vegetative cycle, the holin protein acts to allow release of the accumulated endolysin to the cell wall. Rapid degradation of the peptidoglycan ensues and macroscopic lysis occurs, usually in a characteristically abrupt fashion (FEMS Microbiology Reviews 17 (1995) 191-205).

The holin and endolysin gene, respectively, can be identified in any phage by sequence gazing and verification with a functional test: Computer programs can be used which identify putative endolysins based on their hormology with known and characterized phage endolysins. Holin genes may be identified based on their common architecture which features transmembrane domains sepa-

rated by β-turns as proposed by Steiner et al. (1993) J. Baceriol. 175:1038-1042, which is incorporated herein by reference.

Advantageously the kil-gene is a gene which codes for a nuclease. Thereby, phages are provided which attack the DNA and RNA, respectively, of the bacterial cell thereby rendering the bacteria non-viable.

Preferably, the kil-gene is a gene which codes for a restrictionendonuclease. The restrictionendonuclease is an enzyme which recognizes a specific sequence in a DNA and which cuts the DNA at the recognized region. Many restrictionendonucleases have been identified, whereby an endonuclease is specific for one bacteria, which means that it recognizes DNA which does not naturally occur in the bacteria and thereby digests this foreign DNA. Of course it is necessary that the enzyme does not recognize the phage DNA.

Preferably the kil-gene is a gene which codes for a toxin. In the scope of the present invention the term "toxin" comprises any product which poisens the cell (metabolism). Such toxin genes may be derived from proteic plasmid stabilizations systems, e.g. the doc gene of P, as well as of other post segregational killing systems, e.g. hok of plasmid R1 or gef of E. coli K12 (Poulsen L.K., Larsen N.W., Molin S. and Andersson P. (1984) Molec. Microbiol.. 3:1463-1472; Jensen R.B. and Gerdes K. (1995), Molec. Microbiol. 17:205-210). Many other toxins are well known in the art and any suitable toxin may be applied which attacks the bacteria.

According to an advantageous embodiment of the present invention the phage comprises a non-replicative modification in its genome. The term "non-replicative modification" according to the present invention comprises any modification in the phage genome whereby such a modified phage does not replicate. The modification may be any mutation or deletion as described above in a sequence of the genome which is necessary for the phage replication, e.g. a gene which codes for a protein necessary for replication or a sequence in the genome which regulates the replication of the phage. Thus a phage is provided which infects bacterial cells, causes cell

death without cell lysis, however, which does not replicate and therefore is biologically contained. Thereby a very safe system is provided for killing bacteria without causing danger to other bacterial cells in the environment.

Preferably, a non-replicative modification is present in at least one of the phages genes which code for proteins required for DNA replication. By disrupting the DNA replication of the phage the expression of proteins is disrupted so that no further phages are produced.

Preferably, the non-replicative modification is present in at least one of the phages genes which code for proteins required for phage maturation. Thus, once the phages have infected a bacterial cell, the phage DNA is replicated. However, one of the proteins required for phage maturation is not expressed. Thereby, parts of phages are produced in the bacterial cell, however mature phages are not produced so that no further infection by the phages will occur.

Advantageously the non-replicative modification is present in at least one of its genes which code for coat/capsid proteins. These proteins are necessary for the assembly to a mature phage. If these proteins are not present a mature phage will not be produced whereby such phages comprising the non-replicative modification in a coat/capsid gene will infect a bacterial cell however will not propagate.

A further aspect of the present invention is a method for producing a phage as described above, whereby
- its lysis cassette is non-lytically modified,
- a kil-gene is entered into a non-essential region of the phage genome,
- the modified phage genome is entered into cells of a host strain,
- the modified phages are propagated in the host strain, after which
- the propagated phages are harvested.

The above mentioned definitions are to be applied also for this

aspect of the invention.

The modification of the lysis cassette can be achieved by any known method for entering mutations in genes, e.g. site directed mutation. The respective genes can be identified as mentioned above by sequence gazing and verification by a functional test.

Also the methods for entering a gene into a genome are well known in the art whereby preferably the kil-gene will be entered into a specific sequence of the phage genome using specific endonucleases and ligases.

The modified phage genome is entered into a host strain in order to propagate. Also these methods are well known in the state of the art and any appropriate method may be applied, e.g. electroporation, transfections with liposomes, $CaCl_2$ etc. Once the genome is entered into the host strain, which will preferably be an appropriate bacterial cell, phages will propagate comprising the modified genome. These phages are then isolated and purified from the bacteria and can be stored appropriately according to methods well known in the art, e.g. at 4°C or lyophilized depending on the storage time.

According to an advantageous embodiment of the present invention as the kil-gene a gene coding for a restrictionendonuclease is entered into the phage genome and the modified phage genome is entered into cells of a host strain which expresses the corresponding methylase. As mentioned above restrictoendonucleases are specific to a given species of bacteria and recognize DNA which is foreign to the bacterium. The method of recognition is due to a bacterium specific methylase which is an enzyme which methylates the bacteria DNA in a way which is specific to that bacteria. Any DNA which enters the bacteria does usually not comprise this specific methylation and is therefore recognized by the restrictionendonuclease also specific for that bacteria. A phage comprising a gene coding for a restrictionendonuclease can be entered into any host strain which expresses the corresponding methylase without killing the host strain, since the restrictionendonuclease will not recognize the host strain DNA as foreign DNA and therefore not digest it.

Preferably a gene which codes for the corresponding methylase is cloned into the cells of the host strain. Methods for cloning genes into cells are well known in the art. By cloning the methylase gene into the host strain, any host strain can be used for the propagation of the modified phage since the restrictionendonuclease will not recognize the host strain DNA as foreign DNA and therefore not digest it. With this method according to the present invention it is possible to design any specific phage-host strain system.

Since the propagation conditions as described above do not impose any selective pressure on the phage containing the restrictionendonuclease gene the modified phage is maintained genetically stable over many, e.g more than 5, preferably 10, most preferred 20, generations. This can otherwise be a problem when the modified phage is equiped with the kil-gene, the expression of which is not tightly controlled. Since the selective pressure operating during the propagation will generate less effective progeny.

According to a preferred embodiment of the present invention the phage genome is non-replicatively modified. The term "non-replicatively modified" is the same as defined above for the "non-replicative modification" whereby methods for modifying DNA, e.g. mutating and deleting DNA, respectively, are well known in the art.

Preferably, the non-replicative modification is carried out by expression-disruptingly modifying at least one of the phages genes which code for proteins required for DNA replication and for phage maturation, respectively. Still preferred as the gene for phage maturation a gene which codes for a coat/capsid protein is modified.

Advantageously the wild-type gene which corresponds to the expression-disruptingly modified gene in the phage genome is cloned into the host strain. Thus, a host strain is provided which comprises the gene which is modified in the phage genome. If the non-replicative phage genome is entered into such a host strain comprising the wild-type gene the phage is able to replicate in

that host strain. Thus, a system is provided in which the modified phages according to the present invention are cultivated in a designed host strain. However, as soon as the modified phages are isolated and purified from the designed host strain they are not able to replicate. This method provides a very safe way for producing phages as well as a safe way for using phages against bacteria infections.

A further adventageous embodiment according to the present invention is characterized in that a plasmid comprising both the gene which codes for the corresponding methylase and the gene which codes for the wild-type gene which corresponds to the expression-disruptingly modified gene in the phage genome is cloned into the host strain. This method is a very simple way for providing designed host strains for cultivating the modified phages. Plasmids are well known in the art and any suitable plasmid can be applied. By genetical engineering the gene which codes for the corresponding methylase and the gene which codes for the wild-type gene which corresponds to the expression-disrupting modified gene in a phage genome are entered into the plasmid, e.g. with specific endonucleases/ligases, and the plasmid is entered into the host strain, e.g. via electroporation, liposomes, $CaCl_2$ etc. Host strains which do not comprise this plasmid will comprise DNA which is recognized by the restrictionendonuclease of the phage as foreign DNA and therefore the host strain DNA will be digested once it comprises the phage genome. Furthermore, the phage will not propagate in host strains not comprising the plasmid, since the phage genome comprises an expression-disruptingly modified gene and is therefore not able to replicate without a cell comprising the corresponding wild-type gene. This system is particularly safe and useful for selective propagation.

Another aspect of the present invention is a phage DNA whereby the lysis cassette comprises a non-lytic modification and a non essential region of the DNA comprises at least one kil-gene. The phage DNA is DNA which is modified by genetical engineering as described above. However, the DNA is preferably isolated from phages which have been cultivated, harvested and purified from the host strains as described above. The DNA is easy to handle and can be stored over a long period of time and when necessary

entered into host strain cells in order to produce phages which can be used to treat bacteria infections. The term "phage DNA" according to the present invention comprises the phage genome. However, it may also comprise only a part of the phage genome which is necessary in order to produce phages according to the present invention. Also the phage DNA may be one or more plasmides. The phage DNA may comprise the phage genes in an order in which it naturally occurs in phages. However, the phage genes may also be cloned into a plasmid in a different non-natural order. Furthermore, the phage DNA may comprise additional sequences and genes which do not naturally occur in the phage genome. Preferably, the non-lytic modification in the lysis cassette comprises an expression-disrupting modification in a holin gene.

Still preferred the non-lytic modification in the lysis cassette comprises an expression-disrupting modification in an endolysin gene.

According to an advantageous embodiment of the present invention the kil-gene is a gene which codes for a nuclease. Preferably, the kil-gene is a gene which codes for a restrictionendonuclease. Still preferred, the kil-gene is a gene which codes for a toxin.

Also preferred the phage DNA comprises a non-replicative modification. Advantageously, the non replicative modification is present in at least one of its genes which code for proteins required for DNA replication, in at least one of its genes which code for proteins required for phage maturation, and in at least one of its genes which code for coat/capsid proteins, respectively.

A further aspect of the present invention is the use of the phage as an antibacterial agent. This agent can be used in organisms as well as in any non-living substance, e.g. in a selection medium for cultivation, in any liquids, creams and substances which must be free from bacteria. The agent may of course comprise any further bactericidal, virucial, etc. agents, e.g. antibiotics, chemical substances, etc.

According to a further aspect of the present invention a pharmaceutical preparation is provided which comprises phage according

to the present invention as described above. Also the pharmaceutical preparation may comprise any further antibacterial substance, e.g. antibiotics, virucidal substance, and others.

Preferably, the pharmaceutical preparation further comprises a pharmaceutically acceptable carrier.

The pharmaceutical preparation according to the present invention can be used as a safe treatment of bacterial infections in organisms. Since the bacterial cells do not lyse any side effects of conventional phage therapy are avoided. Furthermore, any side effects resulting from conventional antibiotic treatment are also avoided. This pharmaceutical preparation is particularly useful for the treatment of bacteria which are resistant to antibiotics. The pharmaceutical preparation may be used for example in a treatment against Staphylococcus aureus infections.

According to a further aspect the present invention provides a method for treatment of bacterial infections in an organism whereby a pharmaceutical preparation according to the present invention as described above is administered.

The present invention is described in more detail with the help of the following examples and the annexed figures, whereby

fig.1 shows the strategy for the production of a phage according to the present invention, and

fig. 2 shows the destruction of bacterial cells infected with phage over a time period.

Examples

Example 1: Construction of a phage containing a restrictionendonuclease gene (s. Fig.1)

The restrictionendonuclease II gene of Bacillus subtilis RUB562 (Bacillus globigii) was amplified by PCR using chromosomal DNA. The 5'primer was designed to incorporate the ribosome binding site of the BglII gene and to introduce a BamHI restriction site. The 3'primer included a HindIII-restriction site. The resulting PCR fragment was cloned into the BamHI/HindIII site of the phage vector M13mp18 under transcriptional control of the lac promoter (M13-BglIIR).

Using an analogous strategy the BglII methylase gene (BglIIM) was cloned into the E. coli-P. aeruginosa shuttle vector pUCP24. The M13-BglIIR phage was propagated on E. coli MC4100F' containing plasmid pUCP24BglIIM in LB broth containing 30 µg/ml tetracycline, 15 µg/ml gentamycin and 3 mM IPTG (s. A in Fig.1). Overnight cultures were centrifuged at 6000 g for 5 min and the supernatant was passed twice through a 0.20 µm filter.

The progeny phage was used to infect wt bacteria (s. B in Fig.1). Host DNA was degraded by the enzyme (s. C in Fig.1). The bacteria remain structurally intact for several hours.

Example 2: Efficacy of host cell killing (s. Fig. 2)

The killing efficacy of the M13-BglIIR phage was assessed on E. coli strain MC4100F'. The culture was grown in 10 ml LB broth containing 30 µg/ml tetracycline and 3 mM IPTG at 37°C. The culture was grown to an OD600 of 0.2 and infected with a MOI (multiplicity of infection) of 2. To determine the CFU (colony forming units) of the culture upon phage infection, samples were withdrawn at t=0, 60, 120, 180, 300 and 900 min. These aliquots were used for total cell count measurements and CFU counts on LB plates containing 30 µg/ml tetracycline.

The diagram in Fig.2 shows the colony forming units (■), total cells (♦) and the OD600 (Δ), whereby at t=0 the amount of colony forming units and total cells are over 10^8/ml, at t=900 the

amount of total cells is still over 10^8/ml, the amount of colony forming units is 0.

claims

1. A phage, characterized in that its lysis cassette comprises a non-lytic modification and a non essential region of its genome comprises at least one kil-gene.
2. The phage according to claim 1, characterized in that the non-lytic modification in the lysis cassette comprises an expression-disrupting modification in a holin gene.
3. The phage according to claim 1 or 2, characterized in that the non-lytic modification in the lysis cassette comprises an expression-disrupting modification in an endolysin gene.
4. The phage according to any one of claims 1 to 3, characterized in that the kil-gene is a gene which codes for a nuclease.
5. The phage according to claim 4, characterized in that the kil-gene is a gene which codes for a restrictionendonuclease.
6. The phage according to any one of claims 1 to 3, characterized in that the kil-gene is a gene which codes for a toxin.
7. The phage according to any one of claims 1 to 6, characterized in that it comprises a non-replicative modification in its genome.
8. The phage according to claim 7, characterized in that the non-replicative modification is present in at least one of its genes which code for proteins required for DNA replication.
9. The phage according to claim 7 or 8, characterized in that the non-replicative modification is present in at least one of its genes which code for proteins required for phage maturation.
10. The phage according to claim 9, characterized in that the non-replicative modification is present in at least one of its genes which code for coat/capsid proteins.
11. A method for producing a phage according to any one of claims 1 to 10, characterized in that
- its lysis cassette is non-lytically modified,
- a kil-gene is entered into a non-essential region of the phage genome,
- the modified phage genome is entered into cells of a host strain,
- the modified phages are propagated in the host strain, after which
- the propagated phages are harvested.

12. The method according to claim 11, characterized in that as the kil-gene a gene coding for a restrictionendonuclease is entered into the phage genome and in that the modified phage genome is entered into cells of a host strain which expresses the corresponding methylase.

13. The method according to claim 12, characterized in that a gene which codes for the corresponding methylase is cloned into the cells of the host strain.

14. The method according to any one of claims 11 to 13, characterized in that the phage genome is non-replicatively modified.

15. The method according to claim 14, characterized in that the non-replicative modification is carried out by expression-disruptingly modifying at least one of the phages genes which code for proteins required for DNA replication and for phage maturation, respectively.

16. The method according to claim 15, characterized in that as the gene for phage maturation a gene which codes for a coat/capsid protein is modified.

17. The method according to claim 15 or 16, characterized in that the wild-type gene which corresponds to the expression-disruptingly modified gene in the phage genome is cloned into the host strain.

18. The method according to claims 13 and 17, characterized in that a plasmid comprising both the gene which codes for the corresponding methylase and the gene which codes for the wild-type gene which corresponds to the expression-disruptingly modified gene in the phage genome is cloned into the host strain.

19. A phage DNA, characterized in that the lysis cassette comprises a non-lytic modification and a non essential region of the DNA comprises at least one kil-gene.

20. The phage DNA according to claim 19, characterized in that the non-lytic modification in the lysis cassette comprises an expression-disrupting modification in a holin gene.

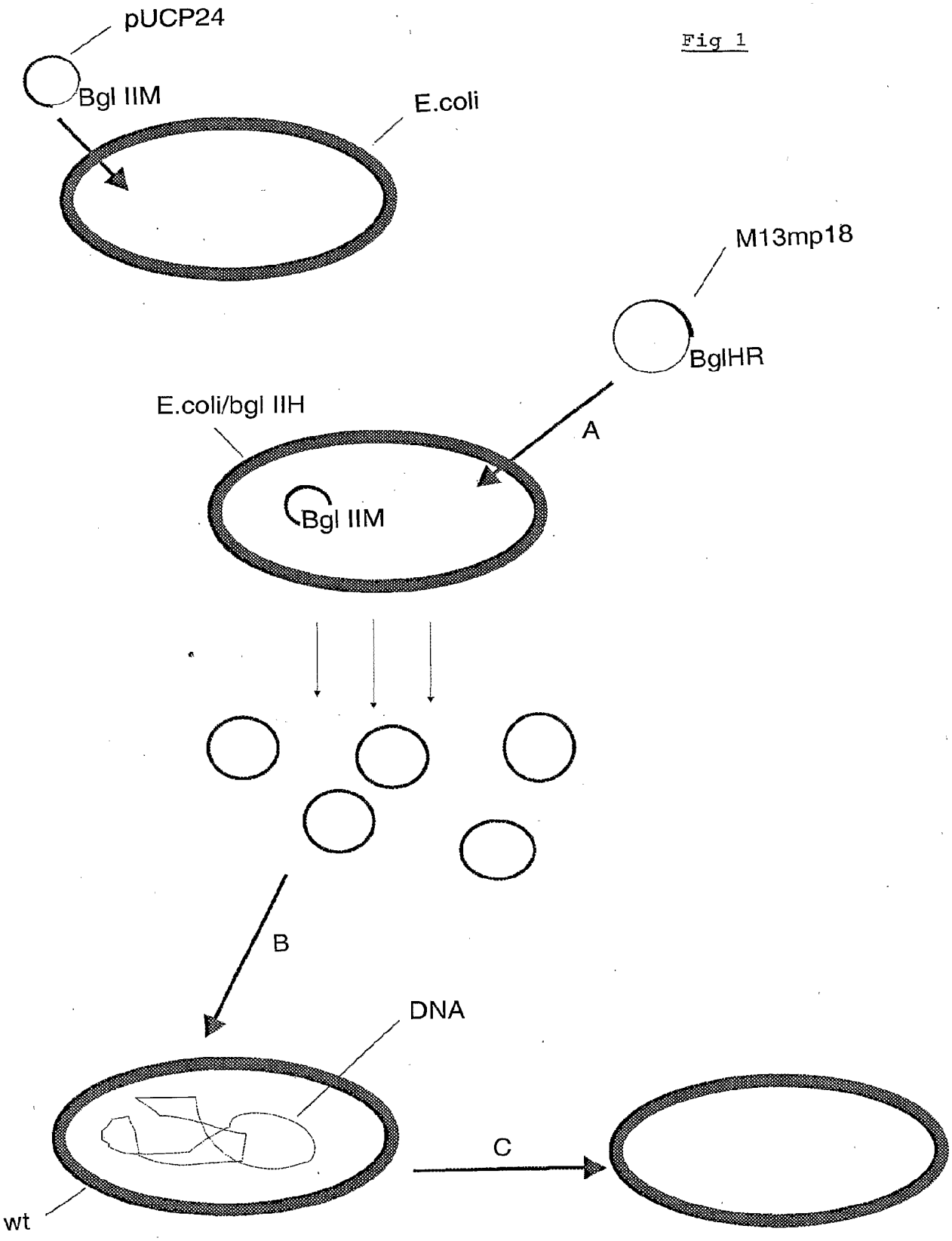
21. The phage DNA according to claim 19 or 20, characterized in that the non-lytic modification in the lysis cassette comprises an expression-disrupting modification in an endolysin gene.

22. The phage DNA according to any one of claims 19 to 21, characterized in that the kil-gene is a gene which codes for a nuclease.

23. The phage DNA according to claim 22, characterized in that

the kil-gene is a gene which codes for a restrictionendonuclease.
24. The phage DNA according to any one of claims 19 to 21, characterized in that the kil-gene is a gene which codes for a toxin.
25. The phage DNA according to any one of claims 19 to 24, characterized in that it comprises a non-replicative modification.
26. The phage DNA according to claim 25, characterized in that the non-replicative modification is present in at least one of its genes which code for proteins required for DNA replication.
27. The phage DNA according to claim 25 or 26, characterized in that the non-replicative modification is present in at least one of its genes which code for proteins required for phage maturation.
28. The phage DNA according to claim 27, characterized in that the non-replicative modification is present in at least one of its genes which code for coat/capsid proteins.
29. Use of the phage according to any one of claims 1 to 10 as an antibacterial agent.
30. Pharmaceutical preparation comprising the phage according to any one of claims 1 to 10.
31. Pharmaceutical preparation according to claim 30 characterized in that it further comprises a pharmaceutically acceptable carrier.

Fig 1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Bgl IIM
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C
wt

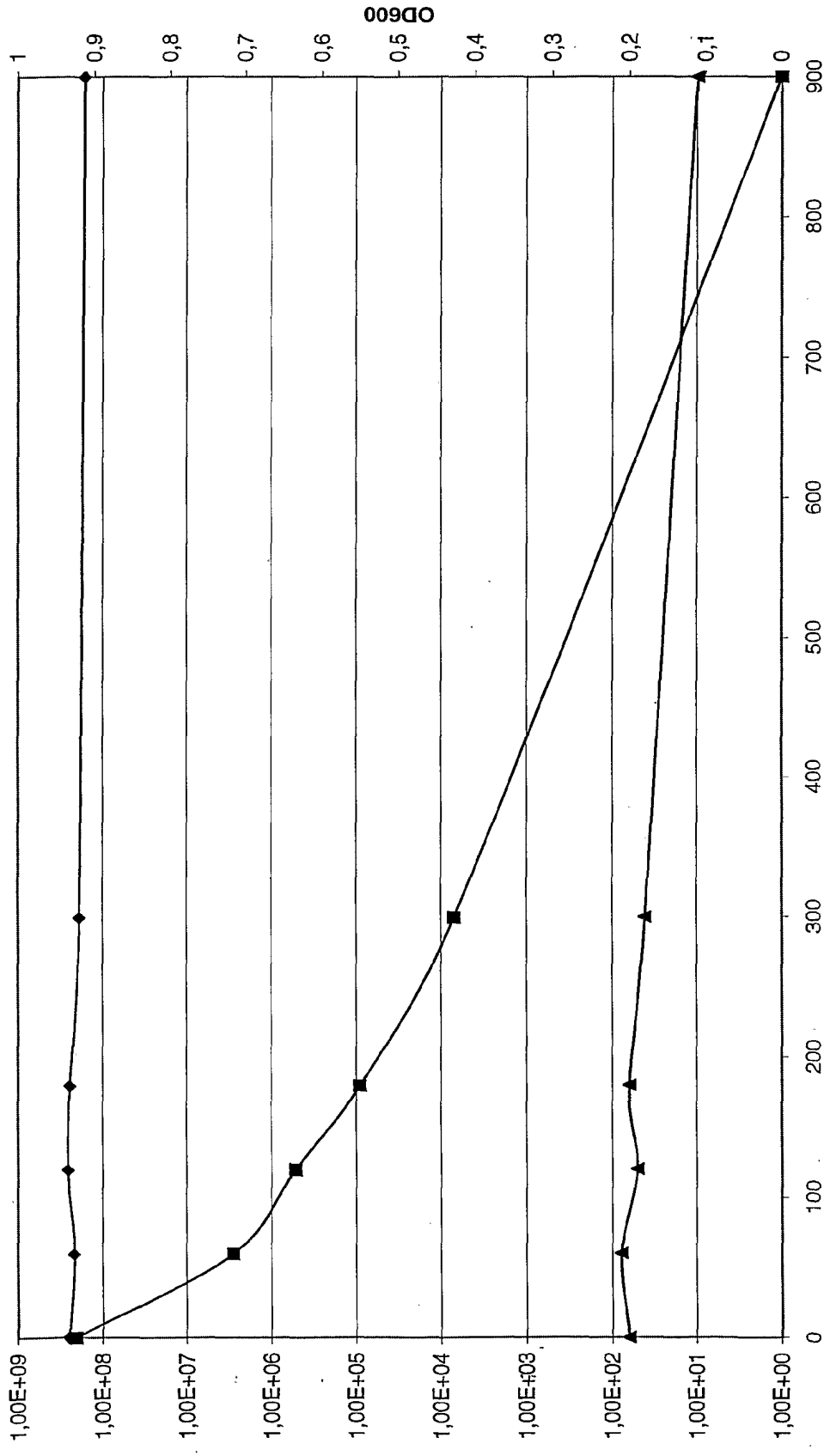
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Fig. 2

INTERNATIONAL SEARCH REPORT

Inter ıal Application No
PC[illegible]P 01

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N7/00 A61K35/76

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, MEDLINE, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CAO JUN ET AL: "Helicobacter pylori-antigen-binding fragments expressed on the filamentous M13 phage prevent bacterial growth." BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1474, no. 1, 6 March 2000 (2000-03-06), pages 107-113, XP002190282 ISSN: 0006-3002 page 109, left-hand column -page 110, left-hand column, paragraph 2 page 111, left-hand column, paragraph 2 -page 112, left-hand column, paragraph 1; figures 4,5 --- -/--	1,11,19, 29,30

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
14 February 2002	11/03/2002

Name and mailing address of the ISA	Authorized officer
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